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# Proteolytic cleavage of the cell surface protein p160 is required for detachment of the fertilization envelope in the sea urchin

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**Abstract**

Sea urchin eggs secrete a serine protease activity, CGSP1, at fertilization that is essential for the block to polyspermy. Several targets of this proteolytic activity on the plasma membrane were identified here using a cell surface biotinylation approach. Amino acid microsequencing of one of these proteins led to the identification of a 4.75-kb cDNA clone from a *Strongylocentrotus purpuratus* ovary cDNA library that encodes a 160-kDa protein called p160. This protein contains five CUB domains and a putative transmembrane domain suggesting that p160 is an integral membrane protein with protein–protein interaction motifs facing the extracellular matrix of the egg. Whole-mount immunolocalization studies demonstrate that p160 is on the surface of the egg, enriched at the tips of microvilli. The protein is removed at fertilization in a protease-dependent manner, and functional assays suggest that p160 serves to link the plasma membrane to the vitelline layer until fertilization. Thus, p160 is a key candidate for a vitelline-layer linker protein, the selective proteolysis of which functions in the block to polyspermy in the sea urchin egg.


*Keywords:* Block to polyspermy; Egg; Fertilization; Protease; Sea urchin; *Strongylocentrotus purpuratus*; Vitelline layer; CGSP1

## Introduction

Fertilization in many animals leads to a rapid transformation of the egg cell surface. Much of this change results from the secretion of cortical granules, responding to the calcium wave in the cytoplasm of the egg. Cortical granules are specialized organelles of the oocyte that modify both the plasma membrane and the extracellular matrix of the egg that results in blocking additional sperm from reaching or fusing with the inseminated egg. Multiple sperm fusions, or polyspermy, are usually lethal in animals due to the addition of supernumerary centrioles and chromosomes. In sea urchins, the surface transformation of the egg is readily apparent in the formation of the fertilization envelope (FE). The FE forms from an interaction of contents exocytosed from the cortical granule with the vitelline layer on the cell surface to create the tough, macromolecular aggregate that is completely partitioned from the newly fertilized egg minutes after insemination (Shapiro et al., 1989). The FE then acts as the permanent block to polyspermy and also provides a protected environment for the embryo as it develops.

Proteolytic activity participates in the modification of the egg cell surface at fertilization in most animals studied, such as mice, frogs, and sea urchins (Shapiro et al., 1989; Wessel et al., 2001). In the sea urchin, proteins that connect the egg vitelline layer to the plasma membrane are hydrolyzed to initiate FE separation from the egg (Mozingo and Chandler, 1991). The severing of connections is caused at least in part by a trypsin-like protease activity that is secreted from the cortical granules following insemination (Alliegro and Schuel, 1985; Carroll and Epel, 1975; Vacquier et al., 1972a). When sea urchin eggs are fertilized in the presence of serine protease inhibitors, the FE remains partially attached to the membrane (Hagström, 1956; Schuel et al., 1973; Vacquier et al., 1972b) and the eggs become polyspermic. Carroll and Epel (1975) isolated a serine protease activity from cortical granule exudate that was described as vitelline delaminase, because it cleaved the connections between the plasma membrane and the vitelline layer.

*Abbreviations:* SBTI, soybean trypsin inhibitor; FE, fertilization envelope; ASW, artificial sea water; DTT, dithiothreitol; CGSP1, cortical granule serine protease; 3-AT, 3-amino-1,2,4-triazole.

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Subsequently, a delaminase activity at 35 kDa was isolated from sea urchin cortical granules (Alliegro and Schuel, 1988). It is now clear that a single serine protease, CGSP1, is stored in the cortical granules and is secreted at fertilization (Haley and Wessel, 1999). CGSP1 autocatalyzes from its 61-kDa proform to 35, 30, and 25 kDa activities that are responsible for its multiple functions at fertilization, many changes of which have been captured by electron microscopic analysis (Mozingo and Chandler, 1991).

Although the cell surface proteins targeted by the protease activity have not been identified, it is known that several are cleaved at fertilization. Shapiro (1975) surface labeled unfertilized *Strongylocentrotus purpuratus* eggs with $^{125}$I and observed that after fertilization, the labeled proteins shifted to a lower molecular weight. This downward shift was prevented when soybean trypsin inhibitor (SBTI) was present, supporting the theory that limited proteolysis of cell surface proteins takes place at insemination. At least 10 labeled polypeptides ranging in size from 22 to 200 kDa are removed from the egg surface after fertilization, constituting up to 25% of the cell surface label (Carroll and Epel, 1981; Johnson and Epel, 1975). These observations suggest that select cell surface proteins participate in the permanent block to polyspermy and are potential targets for the cortical granule protease.

Only a few proteins have been identified that are integral to the egg plasma membrane. In mammalian eggs, a variety of α and β integrins have been described (Almeida et al., 1995; Evans et al., 1995; He et al., 2003; Tarone et al., 1993), as well as several integrin-associated tetraspanins, CD9, CD81, and CD151 (Andria et al., 1992; Chen et al., 1999; Le Naour et al., 2000; Neilson et al., 2000). The function of these proteins is not yet clear, and it is not known if any of these are proteolyzed at fertilization. In the sea urchin, an αBβC integrin has been shown to be present in both cortical granules and on the plasma membrane (Burke et al., 2004; Murray et al., 2000). Upon fertilization, this integrin is removed from the sea urchin egg cell surface and is incorporated into the FE. The αBβC integrin may be a protease target, as its removal from the cell surface is incomplete when eggs are fertilized in SBTI. In the vitelline layer, the egg receptor for sperm is also removed in a protease-dependent fashion (Hirohashi and Lennarz, 1998), which prevents the additional binding to, and fusion with, the fertilized egg.

In this report, we describe a putative vitelline layer linker protein, p160, on the sea urchin egg. This protein has multiple CUB motifs that have been shown in other molecules to function in protein–protein interactions (Bork and Beckmann, 1993). At fertilization, p160 is removed from the egg surface in a protease-dependent manner and if removal is selectively blocked, the separation between the plasma membrane and the newly forming FE is incomplete, much like the phenotype of protease-deficient eggs. The data suggest that this protein serves to attach the vitelline layer to the plasma membrane until insemination, when it is cleaved to allow FE separation from the fertilized egg.

## Materials and methods

### *Animals and reagents*

Adult *Strongylocentrotus purpuratus* were obtained from Charles Hollahan (tidalflux@yahoo.com; Santa Barbara, CA). Gametes were obtained by intercoelomic injection with 0.5 M KCl as described (McClay and Matranga, 1986). All reagents were obtained from Sigma-Aldrich (St. Louis, MO) unless otherwise specified.

### *Biotinylation of egg plasma membrane proteins*

To collect proteins released from the egg cell surface at fertilization and exclude content proteins of the cortical granules, *S. purpuratus* eggs were first dejellied and then treated with 10 mM DTT for 10 min (Epel et al., 1970). The eggs were washed twice in artificial sea water (ASW; Aquarium Systems; Mentor, OH) and 5 ml of packed eggs were then biotinylated with 0.5 mg/ml EZ-Link Sulfo-NHS-LC-biotin (Pierce Biochemicals, Rockford, IL) for 30 min on ice followed by three washes of ice-cold ASW plus 2 mg/ml glycine. The eggs were then activated with 10 μg/ml of calcium ionophore A23187 in 50 ml of ASW. In some experiments, soybean trypsin inhibitor (SBTI) was used at 100 μg/ml to inhibit CGSP1 activity, and 1 mM 3-amino-1,2,4-triazole (3-AT) was used to inhibit ovoperoxidase activity. Five minutes after activation, the eggs were removed by centrifugation at 500 × *g* for 2 min and PMSF (1 mM) was added to the supernatant to block further activity of CGSP1. The supernatant was then dialyzed against $dH_2O$ to remove excess salt and concentrated by lyophilization to a final protein concentration of 0.2 mg/ml. One milliliter of the concentrated supernatant was incubated with 20 μl streptavidin-coated agarose beads for 1 h at 4°C. These beads were then resuspended in 2× SDS sample buffer, boiled, and run on a 4–20% gradient gel. The gel was then electroblotted essentially as described (Towbin et al., 1979) and probed with streptavidin conjugated to alkaline phosphatase (diluted 1:300,000). Avidin binding was then detected by BCIP/NBT colorimetric development (Harlow and Lane, 1988).

### *Identification of egg surface targets*

Biotinylated proteins released by the egg were visualized by Coomassie colloidal blue staining. Three bands of interest, of approximately 85, 30, and 25 kDa in size, were excised and subjected to internal amino acid sequencing at the W.M. Keck Foundation Biotechnology Resource Center (New Haven, CT). The full-length

cDNA of p160 was derived from a Lambda ZAP cDNA library from *S. purpuratus* ovary (Laidlaw and Wessel, 1994). The primers used were as follows: A vector primer corresponding to the forward primer of pBluescript, "pBS forward" 5′-GTAAAACGACGGCCAGT-3′ and an antisense, degenerate primer was designed "80BR1" 5′-CKKYICCIGTIARISWRTAIAC-3′ (where I is inosine, K is G/T; Y is C/T; R is A/G, S is C/G, and W is T/A), from the 85-kDa peptide sequence NTLVYSLTGER. PCR products obtained with Taq DNA polymerase (Life Technologies, Gaithersburg, MD) were ligated into the vector pGEM-T easy (Promega Corp., Madison, WI) and DNA sequencing was performed by the macromolecular sequencing facility at Brown University using an ABI 377 prism automated DNA sequencer. To obtain full-length cDNA encoding p160, additional primers were constructed to the sequence obtained from the original 85-kDa fragment and were used to rescreen the library by PCR and by plaque hybridization. Several overlapping clones were sequenced; these data were analyzed by the University of Wisconsin Genetic Computer Group (UWGCG) sequence analysis package (Devereux et al., 1984).

### *Isolation of expressed proteins for polyclonal antibody production*

Two different polyclonal rabbit antibodies were generated to p160. Antibodies were made in two rabbits to the peptide EISIAMPDFDMGR, obtained by internal amino acid sequencing of the 85 kDa, protease-sensitive band. These animals received four boost injections and the antibodies were designated "antipeptide antibody" (Sigma-Genosys, The Woodlands, TX). An additional antibody was raised against bacterially expressed fusion proteins encompassing the majority of p160, amino acid residues 188–1447, and was designated "antipan p160 antibody". To generate the antipan p160 antibody, expression plasmids were constructed in three segments, p160A, B, and C. Each segment was cloned into the expression plasmid pQE30 (Qiagen, Valencia, CA), which incorporates an N-terminal Histidine tag to the expressed protein. Segment A includes the 1st CUB motif of p160 and was produced by PCR using the primers p160A *Bam*HI forward 5′-GGA TCC CTT CCT GAT TTT CAA CTT ACG-3′ and p160A *Bam*HI reverse 5′-GGA TCC CAG ATA GTA TTG TCG ACG G-3′. Segment B includes the 2nd and 3rd CUB motifs of p160 and was generated with the primers p160B *Sac*I forward 5′-GAG CTC ACC GTC GAC AAT ACT ATC TGC-3′ and p160B *Sac*I reverse 5′-GAG CTC AGA CTG TAA ACT GCC ACC ACA-3′. Segment C codes for the 4th and 5th CUB motifs, up to the transmembrane domain, p160C *Bam*HI forward 5′-GGA TCC TCA TCT GCA TTC TAT CCG CAA-3′, and p160C *Bam*HI reverse 5′-GGA TCC ACA GAT GTA GAT CGT CAG CTC-3′.

To generate each protein segment, 50 ml of an overnight culture was added to 500 ml LB. Cultures were grown at 30°C with vigorous shaking until they reached an $OD_{600}$ of 0.6, and were then induced for 3 h with 0.1 mM IPTG. Cells were pelleted and lysed by stirring for 1 h at room temperature in 6 M guandinium hydrochloride buffer at 5 ml per gram wet weight. Lysate was cleared by centrifugation at 10,000 × *g* for 30 min at room temperature and expressed proteins were then purified by nickel column chromatography (Qiagen) following the manufacturer's protocol. Protein fractions containing the expressed proteins were dialyzed against $dH_2O$, lyophilized, and 0.33 mg of each segment was injected into rabbits for polyclonal antibody production, with four boosts on a 3-week cycle.

### *Immunological analysis*

Immunoblot analysis was performed on unlabeled exudates following electroblotting. Exudate samples collected as above were concentrated by lyophilization after dialysis against $dH_2O$, and 5 μg of protein was run per lane. Following electroblotting, the nitrocellulose was blocked for 2 h in 5% BSA in Tris (50 mM, pH 7.6) buffered saline (150 mM NaCl) containing 0.5% Tw-20 (TBSTB). Preimmune serum of each antibody was diluted 5000 times, immune antipeptide serum was diluted 15,000 times, and antipan p160 serum was diluted 10,000 times, all in TBSTB, and incubated on the blot overnight. The blots were then washed several times in TBSTB, and incubated for 2 h with antirabbit secondary antibody (diluted 30,000 times) conjugated to alkaline phosphatase. Alkaline phosphatase activity was detected as above.

Immunoprecipitation analysis was performed also using antipan p160 antibody on biotinylated exudates. Antibodies were first diluted (1000 times) in TBSTB, incubated for 30 min, and cleared by microcentrifugation. Exudate was then added (equating to approximately 50 μl of eggs for each antibody sample) and incubated with rocking for 2 h. Protein-A Sepharose beads were prewashed with TBSTB for 30 min, and were added to the reaction and rocked at room temperature for 2 h. The beads were extensively washed with TBSTB, then TBS alone. Finally, the beads were boiled in SDS-PAGE sample buffer, electrophoresed, and electroblotted as above, and the immunocaptured biotinylated proteins were identified with alkaline phosphatase-labeled avidin as above.

Immunofluorescence localization of p160 was performed on sections of eggs and zygotes that were fixed and processed as previously described (Haley and Wessel, 1999). Primary antipeptide antiserum was diluted 1:25,000 and the secondary antibody (Cy3-conjugated affinity-purified goat antirabbit IgG; Jackson Immunoresearch, West Grove, PA) was diluted 1:200. Epifluorescent and DIC images were visualized using a Zeiss

Axioplan microscope. Control experiments for these immunolabeling protocols include use of preimmune serum and competition of the antipeptide immune serum (diluted 1:25,000) with 20 ng/ml of the p160 peptide used to generate the antibody or with the equal amount of BSA. Following incubation for 30 min at room temperature in an Eppendorf tube, the samples were centrifuged at 14,000 rpm for 5 min at room temperature, and the supernatant was used to label sections of eggs and embryos as described above.

In addition, immunofluorescence localization of p160 was performed on live, unfixed eggs. Antibodies do not cross the membranes of live eggs, so the staining observed takes place solely on the extracellular face of the plasma membrane. Eggs were dejellied by incubation in pH 5.0 ASW for 2 min and then washed three times in pH 8.0 ASW, with or without DTT treatment. The eggs were incubated on ice in p160 antisera (diluted 1:2000) for 30 min, washed three times in ice-cold ASW, and then incubated in secondary antibody (Cy3-conjugated affinity-purified goat antirabbit IgG; Jackson Immunoresearch; diluted 1:200) for 30 min on ice, and washed three times in ice-cold ASW. No significant label was observed with preimmune (diluted 1:2000) or nonrelevant antisera. Epifluorescence and DIC images were visualized using a Zeiss Axioplan microscope and recorded using a Hamamatsu ORCA-ER camera.

For electron microscopic immunolabeling, both unfertilized and freshly fertilized eggs were fixed in 0.5% glutaraldehyde and 5% formaldehyde, embedded in Spurr's resin (Spurr, 1969) and silver–gold sections (approximately 90 nm) were placed on nickel grids, washed twice in TBS supplemented with 1.0 mg/ml glycine and then washed several times in TBS with 10% fetal calf serum (FCS). The eggs were then labeled with antipeptide and antipan p160 antibody at 1:100 or antiCGSP1 antibody at 1:100 in TBS with 10% FCS, for 2 h with gentle inversion at room temperature. The samples were then washed four times with TBS containing 10% FCS. Gold-conjugated, antirabbit secondary antibody (Jackson Immunoresearch) was added at a concentration of 1:30 in TBS with 10% FCS and the samples were incubated for 2 h with gentle inversion. The samples were then washed six times and postfixed with 0.1% glutaraldehyde in TBS, and stained with uranyl acetate and lead citrate. Sections were visualized at 80 keV with a Philips 410 electron microscope, and images were recorded with a Hamamatsu CCD camera using Advanced Microscopy Techniques imaging software (Danvers, MA).

### *Monovalent Fab production*

Antipeptide and antipan p160 antibodies were both purified by ImmunoPure (G) IgG Purification Kit (Pierce). Monovalent Fab antibodies were generated by using the Pierce ImmunoPure Fab Preparation kit. The Fabs were then purified and concentrated to 8 mg/ml for antipan p160 antibody, and 12 mg/ml for antipeptide antibody.

### *p160 functional assay*

To test if p160 is involved in elevation of the fertilization envelope, dejellied, unfertilized eggs were incubated for 20 min on ice with monovalent Fabs at a final concentration of 0.16 mg/ml for antipan p160 Fabs, or 0.2 mg/ml for antipeptide Fabs, whereas protein-A purified preimmune antibodies were added at 1.7 mg/ml, a 10-fold greater concentration than the specific Fabs. The eggs were then washed three times in ice-cold ASW and activated with A23187 (10 μM) or fertilized with sperm (diluted 1:10,000). To directly detect the presence or absence of p160 following activation in these experiments, as well as the inhibition of fertilization envelope detachment, some batches of eggs were also incubated in secondary antibody (Cy3-conjugated affinity-purified goat antirabbit IgG; Jackson Immunoresearch; diluted 1:200) for 30 min on ice, and washed three times in ice-cold ASW before activation. Formation of the fertilization envelope was observed by brightfield and epifluorescence microscopy using a Zeiss Axioplan microscope.

### *Protein isolation*

CGSP1 was purified from unfertilized eggs by SBTI chromatography essentially as described (Alliegro and Schuel, 1985; Fodor et al., 1975; Haley and Wessel, 1999). The protease fractions were checked for purity by Western blot and Coomassie blue staining of SDS-PAGE gels and analyzed for activity with BAEE (Nα-benzoyl-L-arginine ethyl ester, a chromogenic substrate for trypsin-like proteases). To treat eggs with the endogenous CGSP1, the isolated protease was added at 10 μg/ml to 250 μl of washed, dejellied eggs for 5 min on ice. The eggs were then washed three times with cold ASW and labeled as above with antip160 antibody (dilution 1:1000) in 1 ml ASW and visualized by epifluorescence.

Cell surface complex was isolated as described (Crabb and Jackson, 1985), and plasma membrane was isolated as described (Kinsey, 1986). For Western blotting analysis, 5 μg of total protein of each sample was loaded on a 10–20% gradient gel and electroblotted. The blots were then probed with antipan p160 antibody at a dilution of 1:500 and detected with a secondary alkaline phosphatase conjugate (see above).

## Results

### *The release of select egg cell surface proteins at fertilization is protease dependent*

At least nine biotinylated proteins ranging in size from 25 to 150 kDa are released from the egg surface by calcium ionophore activation (Fig. 1) or activation by insemination (data not shown). Supernatants of biotinylated eggs were analyzed from eggs before activation, as

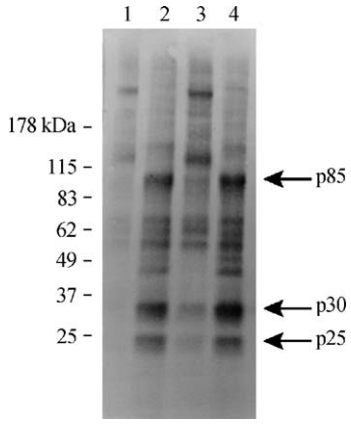


Fig. 1. The release of some egg surface proteins is dependent on serine protease activity. Lane 1, supernatant before activation. Lane 2, supernatant 5 min post activation. Lane 3, supernatant 5 min post activation, containing SBTI. Lane 4, supernatant 5 min post activation, containing 3-AT. Arrows indicate the major bands that are potential protease targets that were here subjected to amino acid sequence.

well as from eggs activated in ASW, in the presence of the soybean trypsin inhibitor (SBTI), or in the presence of the ovoperoxidase inhibitor 3-AT. Two large proteins, at approximately 250 and 125 kDa, are visible in the supernatant before activation (Fig. 1, lane 1), and these proteins were not analyzed, as their release is not solely dependent on protease activity. Other bands are released at egg activation, such as three bands of approximately 62 kDa, but are released independent of protease activity, and thus were also not considered ideal candidates for analysis here. Release of a subpopulation of the biotinylated proteins is blocked when protease activity is inhibited, which suggests that these proteins are biological substrates of CGSP1 (Fig. 1, compare lanes 2 and 3). We focused our attention here on three protein bands at 85, 30, and 25 kDa whose cleavage is apparently protease dependent, and not affected by the presence of 3-AT. Each of these proteins were subjected to internal amino acid sequencing.

Several peptide sequences were obtained from the protease sensitive bands: EISIAMPDFDMGR and NTLVYSLTGER from the 85-kDa protein band, GFQAFYSLQK and STVEGQQFK from the 30-kDa band (Fig. 2), and GFQAFYSLQK was acquired from the 25-kDa band. This latter peptide sequence is identical to one from the 30-kDa band, and suggests that the two fragments originated from the same, larger protein. None of these peptides corresponded with any known proteins in the GenBank and SwissProt databases. Degenerate primers were used to screen a cDNA library constructed from *S. purpuratus* ovaries (Laidlaw and Wessel, 1994). Overlapping cDNA clones of 4749 nucleotides were obtained from this library including a full-length coding region of 4413 nucleotides, encoding an open reading frame of 1471 amino acids, designated p160.

All four peptides obtained from internal amino acid sequencing were found within the p160 cDNA sequence prediction, which demonstrates that the 85-, 30-, and 25-kDa biotinylated bands each originated from the same parent protein. The protein encoded by the full-length cDNA clone is predicted to be 162.8 kDa and does not closely match any other protein in peptide- or nucleotide-based databases in the public domain. From the predicted amino acid sequence, several potential characteristics were determined (Fig. 2). A signal sequence is at its N-terminus between residues 1 and 17, and a predicted transmembrane domain is present near the C-terminus at residues 1444–1463. In addition, five CUB motifs are predicted to form throughout the protein. CUB motifs are hallmarks of extracellular proteins and are thought to function in protein–protein interactions (Bork and Beckmann, 1993).

Three antibodies were generated to the p160 protein, two to a synthetic peptide of the 85-kDa fragment (antipeptide antibody) and another to the majority of the p160 protein produced by heterologous expression in bacteria (antipan p160 antibody). As seen by both immunoblotting and immunoprecipitation, both antibodies selectively bind to proteolytic fragments of the p160 protein released at fertilization (Fig. 3). The antipeptide antibody selectively recognizes an 85-kDa band and a 30-kDa doublet, whereas the antipan p160 antibody, which represents much of the p160 protein, also detects a doublet at 60 kDa. Analysis of the exudate by immunoprecipitation shows that the antipan p160 antibody recognizes the same bands detected by blotting, although in different relative amounts. The different ratios of each polypeptide immunoprecipitated may reflect differences in inherent solubility, differences in accessibility of the antibody as a result of its native configuration, or by complexing to other proteins of the exudate. Immunoprecipitation with the antipeptide antibody was unsuccessful, and may mean that this single site is buried in the native protein, or is interacting with other exudate proteins. Each of the bands immunoprecipitated by the antipan p160 antibody appears to have originated from the parent p160 protein and not a different, coprecipitated protein because they are each detected by both immunoprecipitation (native) and immunoblotting (denatured) techniques. We do not detect any protein at the full-length p160 size predicted by the cDNA sequence, and this likely reflects the presence of the transmembrane domain of the protein remaining in the cell following its cleavage. The full-length p160 size, however, is detected in cell surface fractionations (see below).

### *p160 localizes to the plasma membrane of the unfertilized egg and is removed at fertilization*

As the biotinylation experiments suggested that p160 was on the surface of the egg and cleaved at fertilization, immunolocalizations were performed on sectioned eggs and fertilized embryos to further analyze the localization of the protein. In the unfertilized egg, p160 immunolab-

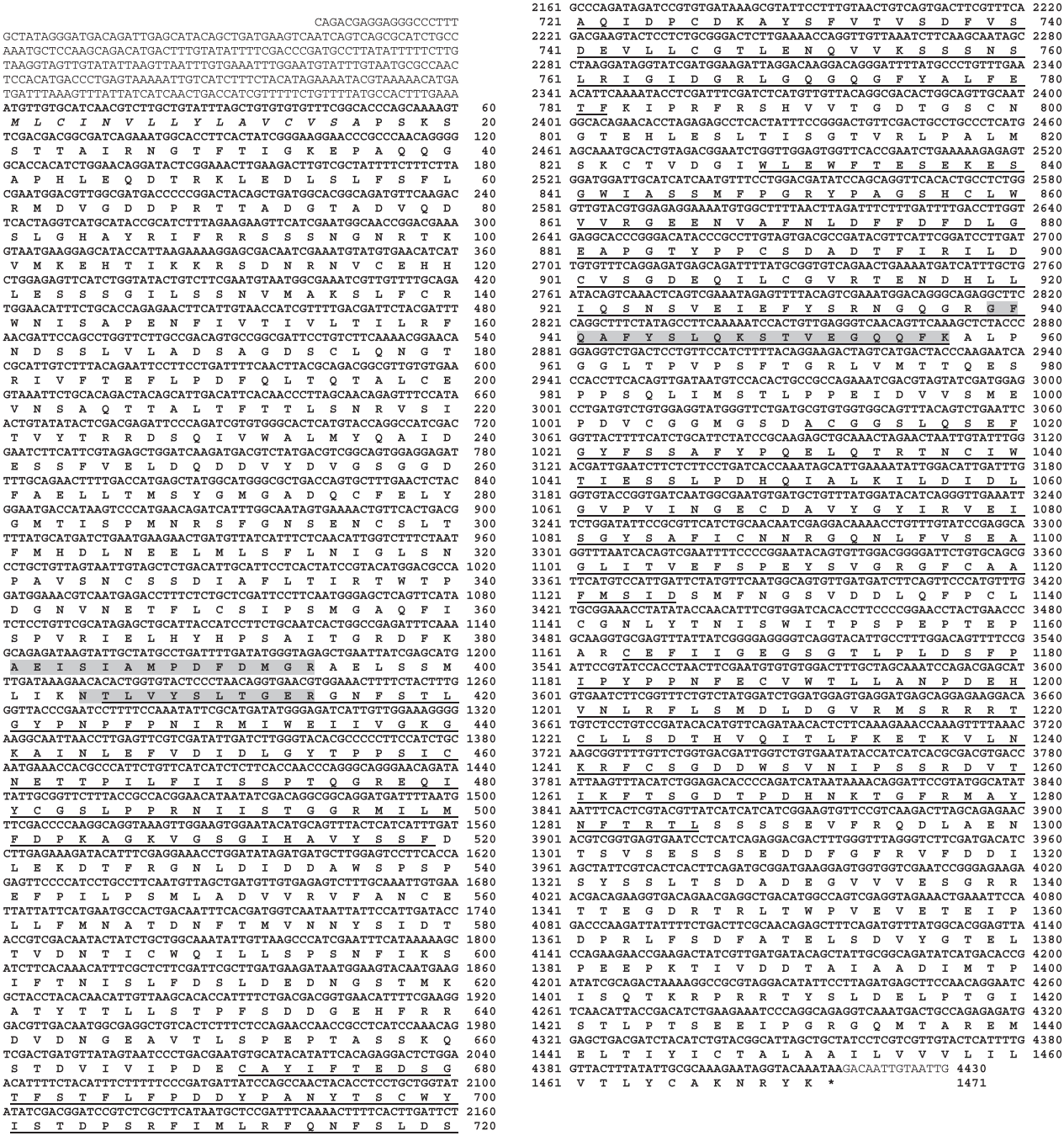

Fig. 2. The amino acid sequence of p160. The signal sequence (amino acid residues 1–17) is in italics, the predicted CUB domains are underlined, and the sequences obtained by internal amino acid sequencing are highlighted. The predicted transmembrane domain encompassed amino acid residues 1444–1463. The GenBank accession number for p160 is AY38618.

eling is evident on the plasma membrane (Figs. 4A,B). However, no significant label is present on the plasma membrane or in the fertilization envelope when sections of zygotes are labeled with the antibody (Figs. 4C,D). Immunolabel signal was specifically competed away in unfertilized egg sections when the antibody was first incubated with the peptide used to generate the antisera (Figs. 4E,F), but not with BSA as a competitor (data not shown).

These results are complemented by electron microscopic immunoanalysis of eggs with both the antipeptide and antipan p160 antibodies. In unfertilized eggs, p160 signal is enriched at the tips of microvilli (Fig. 5; Table 1). Once the eggs are activated, the label immediately disappears from the plasma membrane and transiently remains on the newly forming envelope; after about 2 min, p160 signal is no longer detectable in the envelope. Furthermore, whole-mount immunolocalization studies on

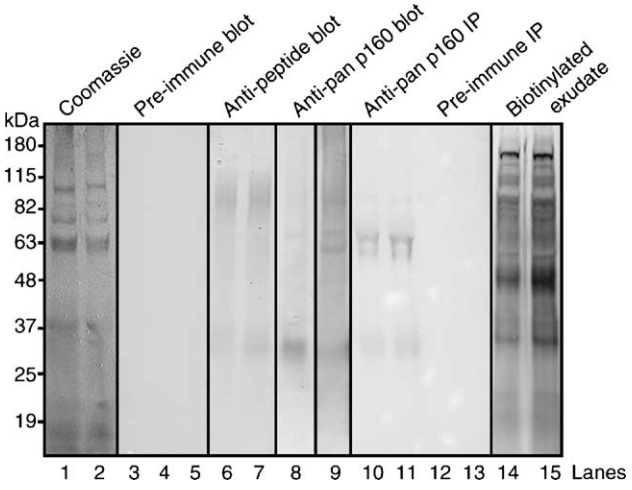


Fig. 3. p160 is proteolyzed and released from the cell surface at fertilization. Two different preparations of exudate released following fertilization were stained with Coomassie (lanes 1, 2). These samples were then immunoblotted against antipeptide preimmune serum (lanes 3–5), antipeptide serum (antipeptide; lanes 6, 7), antipan p160 serum (lanes 8, 9, shown from two different gels). The antibodies detect an overlapping set of p160 fragments at 85, 60, and 35 kDa. Samples were also immunoprecipitated with either antipan p160 (lanes 10, 11) or preimmune serum (lanes 12, 13). The starting samples of two different biotinylated exudates used in the immunoprecipitation experiments are shown (lanes 14, 15). Note that the p160 immunoblot results recapitulate the immunoprecipitation results.

live eggs also demonstrated that p160 is located on the plasma membrane of the unfertilized egg, and is completely removed within 3 min of activation/fertilization

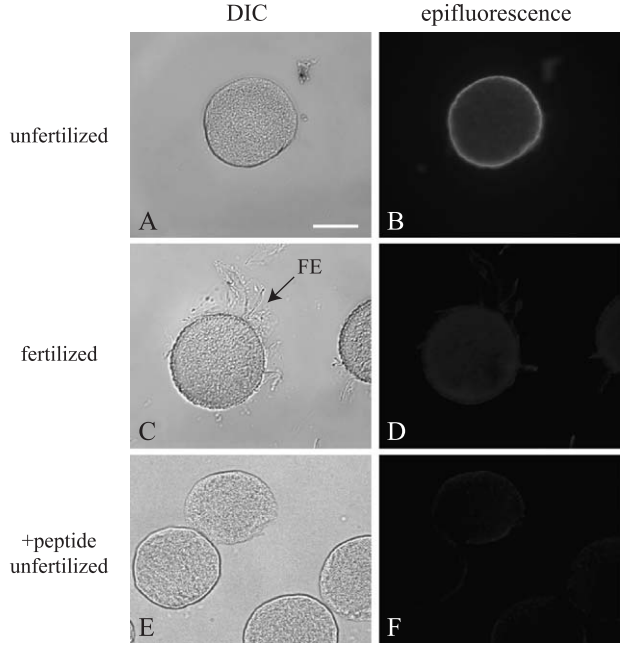


Fig. 4. p160 is on the egg cell surface, and is removed at fertilization. Sections of fixed unfertilized and fertilized eggs were immunolabeled with the antipan p160 antibody. (A, B) Eggs; (C, D) 5 min post fertilization (FE denotes fertilization envelope, which is misshapened during processing); (E, F) immunolabeling of eggs in which the antipeptide antibody was preincubated with the immune peptide. Scale bar = 40 μm.

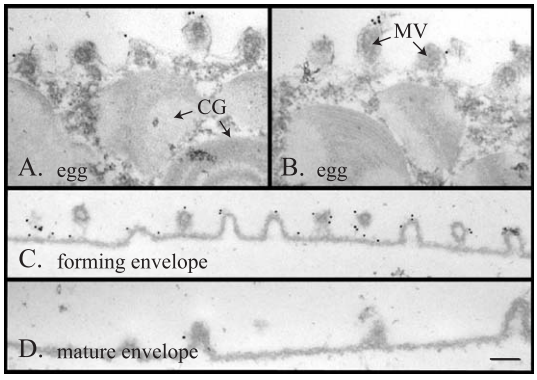


Fig. 5. Ultrastructural immunolocalization of p160 (using the antipeptide antibody) shows its enrichment at the tips of the microvilli in unfertilized eggs (A, B), a transient presence in the forming fertilization envelope (C), and a lack of p160 signal in the mature fertilization envelope (D). CG: cortical granules; MV: microvilli. Scale bar = 0.25 μm.

(Fig. 6). p160 is also not detected in Western blots of mature fertilization envelopes (data not shown).

Cell fractionation studies also supports the hypothesis that p160 is present in the plasma membrane. Equal amounts of whole egg lysate, cell surface complex (comprised primarily of cortical granules and plasma membrane), and purified egg plasma membrane were analyzed by Western blotting using the antipeptide antibody as a probe. A specific band of about 160 kDa is enriched in the plasma membrane samples, corresponding to the predicted size of the protein (Fig. 7). A second, slightly faster migrating band is also present perhaps representing a proteolytic version resulting from the cell fractionation approach. Overall, these studies suggest

Table 1
Quantitation of p160 ultrastructural immunolabeling

| | Particles per micron |
|---|---|
| *Antiprotease* | |
| Egg cell surface | 0.06 ± 0.02 (2) |
| Fertilized egg cell surface | 0 (2) |
| Envelope | 0 (2) |
| *Antipeptide* | |
| Egg cell surface | 1.63 ± 0.55 (4) |
| Fertilized egg cell surface | 0.09 ± 0.03 (3) |
| Forming envelope | 1.80 ± 0.53 (2) |
| Envelope | 0.39 ± 0.14 (3) |
| *Antipan p160* | |
| Egg cell surface | 0.82 ± 0.21 (3) |
| Fertilized egg cell surface | 0.12 ± 0.09 (2) |
| Envelope | 0.36 ± 0.18 (2) |
| *Surface labeling of p160* | |
| Distal one-third of a microvillus | 76% |
| Other surface area labeling | 24% |

*Note.* All values shown are ±1 SD; numbers in parenthesis indicate the number of cells examined. For cells labeled with the antiprotease antibody, at least 12 measurements were taken; for cells labeled with the antipeptide antibody, at least 25 measurements were taken; and for all cells labeled with the antipan p160 antibody, at least eight measurements were taken.

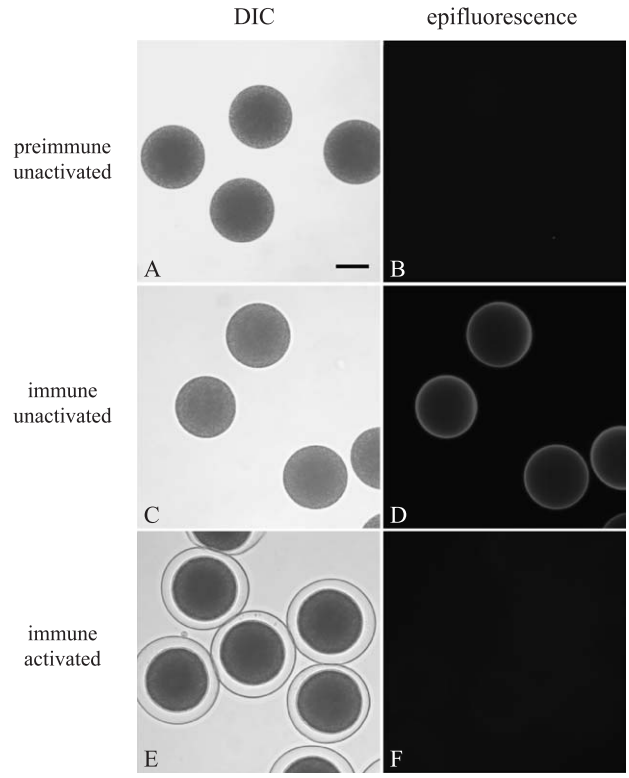


Fig. 6. Live whole-mount analysis demonstrated that p160 is removed from the egg surface upon activation with the calcium ionophore A23187. (A, B) Eggs labeled with preimmune antibody (antipan p160); (C, D) eggs labeled with antipan p160 antibody; (E, F) ionophore activated eggs labeled with antipan p160 antibodies, 3 min post activation. Scale bar = 40 μm.

that p160 is removed from the egg cell surface immediately at fertilization, and it is subsequently degraded, or released from the forming fertilization envelope.

*The removal of p160 from the egg surface is protease dependent, and is required for fertilization envelope elevation*

Chemical reduction of the egg surface by DTT disrupts the vitelline layer and thus a FE does not form following egg activation (Epel et al., 1970; Vacquier et al., 1972b). DTT treatment, however, does not remove p160 on eggs (Figs. 8A,C). Following fertilization or egg activation by calcium ionophore A23187, however, p160 is still proteolyzed from DTT treated eggs, but somewhat slower than in eggs with an intact FE (5 vs. 3 min). Slower removal of the protein in these experiments may be due to the lack of a fertilization envelope, which may serve to concentrate protease activity transiently on the surface of the egg. DTT-treated eggs that were incubated in SBTI during activation did not completely lose p160 label, even after 10 min (Figs. 8B,D). Furthermore, p160 is removed from unfertilized eggs treated with purified CGSP1 (Fig. 9). Collectively, these results suggest that the cortical granule serine protease activity CGSP1 is necessary for p160 removal during fertilization.

To test the function of p160 we used an immunointerference assay. Eggs were incubated in antipan p160 whole antibody or monovalent Fabs of the same, and washed before activation. Immune reagents could potentially block accessibility of the protease to p160 and thereby reveal the function of p160 cleavage. Eggs treated with antipan p160 Fabs demonstrate an abnormal fertilization envelope, much like the phenotype observed when CGSP1 is inhibited (Fig. 10; Haley and Wessel, 1999). One minute following activation in the presence of antipan p160 Fabs, 91% of eggs displayed abnormal or "blebby" envelopes, while 7% exhibited no lifting of the envelope, and 2% had normal envelopes. Three different antibodies prepared in two different ways (two antipeptide antibodies, and one antipan p160 antibody used

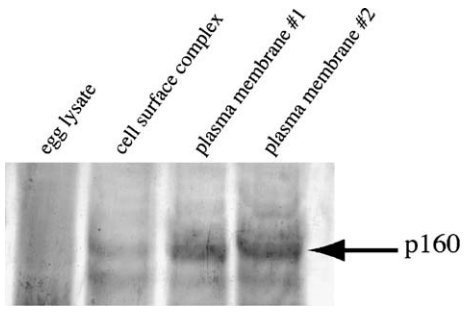


Fig. 7. p160 is enriched in the egg plasma membrane. Immunoblot analyses of different egg fractions probed with antipeptide antibody. Lane 1: whole egg lysate; lane 2: cell surface complex; lanes 3 and 4: different preparations of egg plasma membrane. Five micrograms of each preparation was loaded in each lane. The arrow indicates the band of 160 kDa.

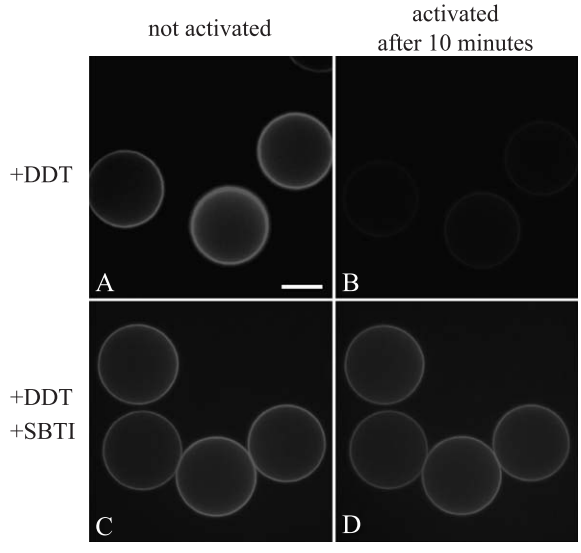


Fig. 8. p160 removal from the egg surface is serine protease dependent. Epifluorescence images of live eggs immunolabeled with the antipeptide antibody: (A) p160-labeled control eggs and (B) after 10 min post activation with A23187. (C) p160-labeled eggs, incubated in SBTI before activation. (D) In the presence of SBTI, p160 label is not removed from activated eggs, up to 10 min post activation. Scale bar = 40 μm.

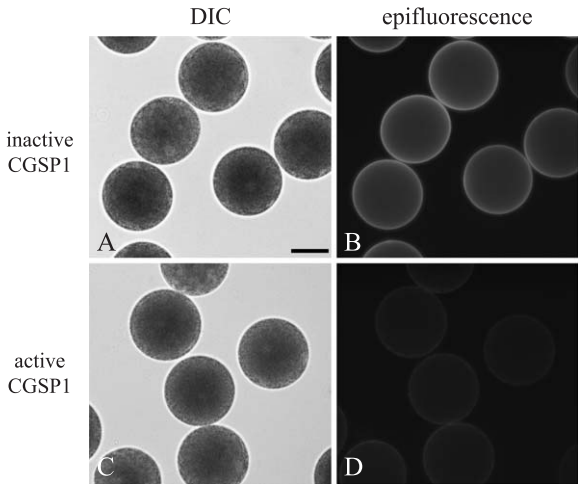


Fig. 9. Purified CGSP1 removes p160 from the egg surface. Live *S. purpuratus* eggs were treated for 5 min with CGSP1 and subsequently labeled with antipan p160 antibody. p160 signal remained on eggs treated with boiled, inactive CGSP1 (A, B), while p160 signal was cleaved from eggs treated with active CGSP1 (C, D). Scale bar = 40 μm.

either as whole, multivalent antibodies or as monovalent, Fabs) gave the same results, while preimmune serum or unrelated immune sera at the same concentrations did not

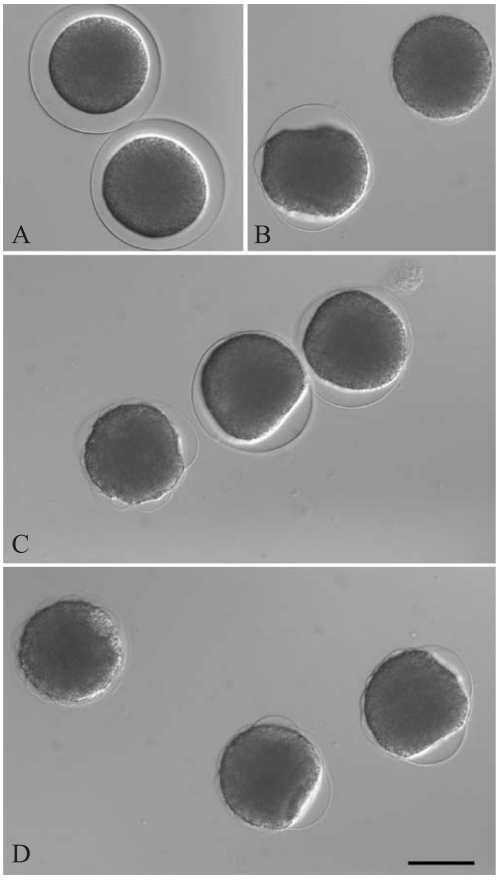


Fig. 10. Fertilization envelope detachment from the plasma membrane is inhibited when live eggs are incubated in antipan p160 Fabs (B, C, D) but not in preimmune sera (A) before activation with A23187. All panels are bright-field images. Scale bar = 40 μm.

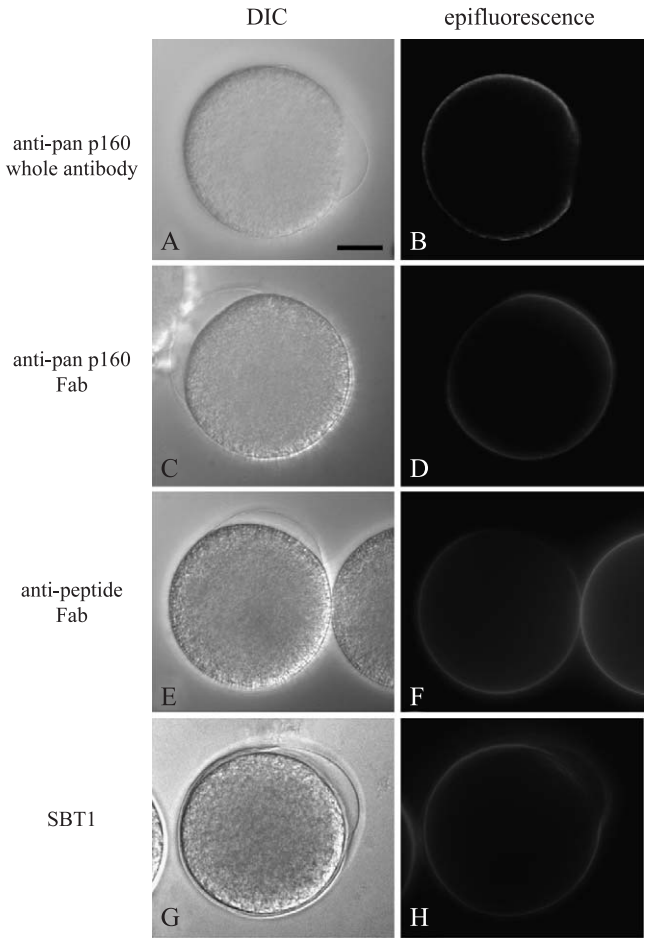


Fig. 11. Proper formation of the fertilization envelope is prevented by either protease inhibitors or by antip160 antibodies upon activation by A23187. (A, B) Antipan p160 whole antibody; (C, D) antipan p160 Fab; (E, F) antipeptide Fab; (G, H) SBTI. Scale bar = 20 μm.

alter proper envelope formation. To detect p160 during egg activation, some batches of eggs were additionally labeled with fluorochrome-conjugated secondary antibody before activation. p160 labeling is observed only where the envelope has not lifted away from the plasma membrane (Fig. 11), which further suggests it is involved in the connections between the plasma membrane and the vitelline layer, and needs to be severed for the envelope to properly separate from the membrane.

## Discussion

Fertilization rapidly transforms the egg surface, resulting in the block to polyspermy. Dramatic morphological changes take place as a result of cortical granule exocytosis to bring about this function, yet relatively few proteins on the plasma membrane of any egg have been characterized, and little is known of their modification following fertilization. To begin to understand the transformation that occurs on the sea urchin egg, we focused here on cell surface proteins that

serve as targets of the cortical granule protease. We observed that at least nine proteins accessible to biotinylation were released from the egg cell surface, ranging in size from approximately 25 to 150 kDa. These results are similar to the results reported previously (Carroll and Epel, 1981; Johnson and Epel, 1975) using surface-radiolabeled eggs. In addition, Shapiro (1975) showed that the release of some of these proteins is blocked by the addition of protease inhibitors, suggesting that selective proteolysis takes place on the egg surface at fertilization. We focused on the proteins that most clearly demonstrated a dependence on serine protease cleavage at fertilization and utilized biotinylation as a detection and purification scheme.

The structure of p160 is composed mainly of five predicted CUB motifs, characterized by regions of approximately 110 amino acids containing conserved cysteines that form disulfide-bonded loops. The function of CUB motifs appears to be in mediating interactions with ligands, with other CUB-containing proteins, or with proteoglycans (Bork and Beckmann, 1993). These motifs are found in many extracellular proteins, including tolloid proteases (Shimell et al., 1991), complement proteases (Journet and Tosi, 1986), spermadhesins (single CUB proteins involved in primary gamete interaction in mammals; Romao et al., 1997), and sea urchin fibropellins I and III, proteins that mediate interactions between migrating cells and the hyaline layer during gastrulation (Burke et al., 1998). It is possible that the CUB domain in p160 is disrupted when the unfertilized eggs are treated with DTT, with subsequent loss of p160-mediated interactions. This disruption could be a contributing factor to the alteration of the vitelline layer upon DTT treatment that results in the absence of an envelope at fertilization.

Several results support the hypothesis that p160 is cleaved at fertilization by the cortical granule protease, CGSP1—(1) the removal of p160 is coincident with cortical granule exocytosis when CGSP1, along with the other cortical granule contents, is secreted; (2) purified CGSP1 hydrolyzes p160 from the egg surface; (3) p160 removal is blocked by serine protease inhibitors at fertilization. CGSP1 also apparently cleaves p160 more than once following fertilization, as multiple proteolytic products are observed.

We propose that p160 is a target substrate of CGSP1 on the egg, the cleavage of which is required for FE detachment from the cell surface. Our initial tests of p160 function support this hypothesis. Previous ultrastructural analysis of the egg surface using quick-freeze, deep-etch, electron microscopy has shown that the vitelline layer contains oblique and horizontal filaments attached to the tips of microvilli that are normally cleaved after fertilization, but remain if the protease is inhibited (Larabell and Chandler, 1991; Mozingo and Chandler, 1991). The molecular identities of the filaments and post proteins have not been determined, but p160 now serves as one viable candidate. The vitelline layer is composed of as many as 25 different proteins (Niman et al., 1984) providing a wealth of interaction potential for p160.

Some proteins are released from the egg surface independent of protease activity. These include a cluster of peptides at approximately 62 kDa, and their release might be dependent on phospholipases or another activity at fertilization and therefore are probably not CGSP1 targets. Also, while two large proteins of approximately 250 and 125 kDa appeared to be enriched in the exudate in the absence of protease activity, they were also present in the supernatant samples of untreated eggs and therefore were not analyzed in this study as proteins removed exclusively by CGSP1 proteolysis. Carroll and Epel (1981) described the release of two bands, at 200 and 185 kDa, from labeled eggs incubated in seawater. It is possible that the difference in sizes from our observation may be due simply to different surface labeling or other experimental conditions. Based on observations from our biotinylation assays, these large proteins may be nonconvalently associated with the cell surface, and then secondarily cleaved following fertilization, as they are not detected in supernatants without protease inhibitors.

It is likely that a variety of surface proteins are cleaved at fertilization. In addition to p160, the protease may also cleave proteins that are not as abundant on the egg surface, are more subtly cleaved and therefore escaped detection, or they may be in the vitelline layer and removed by DTT. The protease may even cleave proteins that participate in cell signaling at fertilization. In the case of starfish eggs, exogenously added proteases stimulate fertilization-like responses (Carroll and Jaffe, 1995). Also, an integrin has been described by Murray et al. (2000) that is removed at fertilization, presumably by protease activity. Like p160, the αBβC integrin may have a protein–protein interaction function that must be removed for proper envelope elevation. The integrin is also localized to the tips of microvilli, as is p160 and a vitelline layer sperm binding protein, which also serves as a potential protease substrate (Hirohashi and Lennarz, 1998; Ohlendieck et al., 1994). Perhaps the microvilli contain domains of plasma membrane proteins different from the rest of the egg cell surface that concentrate proteins functioning in sperm recognition, cell signaling, and a rapid block to polyspermy. In addition, CUB domains are known to interact with carbohydrates (Topfer-Petersen et al., 1998) and p160 possibly binds to carbohydrates in the heavily glycosylated vitelline layer or perhaps in egg jelly. Future experiments will include testing the molecular interactions of p160 with other membrane proteins on the cell surface, as well as those that comprise the vitelline layer.

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